Engineered polyamine accumulation in tomato enhances phytonutrient content, juice quality, and vine life

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Polyamines, ubiquitous organic aliphatic cations, have been implicated in a myriad of physiological and developmental processes in many organisms, but their *in vivo* functions remain to be determined. We expressed a yeast *S*-adenosylmethionine decarboxylase gene (ySAMdc; *Spe2*) fused with a ripening-inducible E8 promoter to specifically increase levels of the polyamines spermidine and spermine in tomato fruit during ripening. Independent transgenic plants and their segregating lines were evaluated after cultivation in the greenhouse and in the field for five successive generations. The enhanced expression of the ySAMdc gene resulted in increased conversion of putrescine into higher polyamines and thus to ripening-specific accumulation of spermidine and spermine. This led to an increase in lycopene, prolonged vine life, and enhanced fruit juice quality. Lycopene levels in cultivated tomatoes are generally low, and increasing them in the fruit enhances its nutrient value. Furthermore, the rates of ethylene production in the transgenic tomato fruit were consistently higher than those in the nontransgenic control fruit. These data show that polyamine and ethylene biosynthesis pathways can act simultaneously in ripening tomato fruit. Taken together, these results provide the first direct evidence for a physiological role of polyamines and demonstrate an approach to improving nutritional quality, juice quality, and vine life of tomato fruit.

Tomato fruit is an important source of phytonutrients and micronutrients and is consumed both fresh and as a processed product. Tomato cultivation and processing is a billion-dollar industry in the US alone[1], with tomatoes ranking close to potatoes among commercial vegetable crops in terms of yield and consumption. The tomato has a relatively small genome[2] and is one of the foremost plant models in the agricultural application of molecular genetics to understanding and controlling fruit ripening[3]. Ripening of tomato fruit involves differentiation of chloroplasts into chromoplasts, which results in the accumulation of nutrients beneficial to human health, such as β-carotene, vitamin E, and lycopene. Careful genetic manipulation of the ripening process should allow development of transgenic plants with enhanced phytonutrient content[4].

Reverse genetics has established that ethylene is the "ripening" hormone in plants[5]. Genetic modification of ethylene biosynthesis and ethylene responsiveness has led to the production of tomato fruit with delayed ripening characteristics and increased shelf life[3,5–8]. Furthermore, the *in vivo* function of several genes involved in fruit development and ripening has been tested via antisense RNA technology or overexpression of candidate gene(s)[3]. Furthering these studies should delineate the roles played by ethylene and other plant growth substances in controlling fruit ripening, flower wilting, leaf senescence, and organ abscission.

A characteristic feature differentiating the growth phase of a fruit from its ripening phase is the inverse developmental relationship of spermidine and spermine accumulation and ethylene production. Polyamines, ubiquitous and abundant in actively growing tissues, generally decrease during fruit ripening and tissue senescence[9,10]. Common polyamines include diamines (putrescine, cadaverine) and higher polyamines (spermidine and spermine). Diamines are synthesized from basic amino acids[10], with putrescine being formed from ornithine by ornithine decarboxylase or from arginine by arginine decarboxylase (Fig. 1). Decarboxylated *S*-adenosylmethionine (SAM), synthesized from SAM by SAM decarboxylase, is used as an aminopropyl-group donor, first by spermidine synthase to form spermidine from putrescine and then by spermine synthase to form spermine from spermidine. Methylthioadenosine (MTA) is a common byproduct. SAM decarboxylase is a rate-limiting enzyme in polyamine biosynthesis[9,10]. SAM is converted by 1-aminocyclopropane-1-carboxylic acid (ACC) synthase to MTA and ACC, an immediate precursor of ethylene[6]. Thus, both ethylene and polyamine biosynthetic pathways use SAM as substrate and produce MTA as a byproduct.

In vitro studies have suggested that polyamines act as anti-senescence and anti-ripening regulators by inhibiting ethylene biosynthesis in fruit and vegetative tissues[10,11] and, conversely, that ethylene inhibits the enzymes in the biosynthesis of polyamines[12]. These studies pointed to the possibility of a temporal relationship between the actions of polyamines and ethylene during plant development, and have led to suggestions that the relative amounts of

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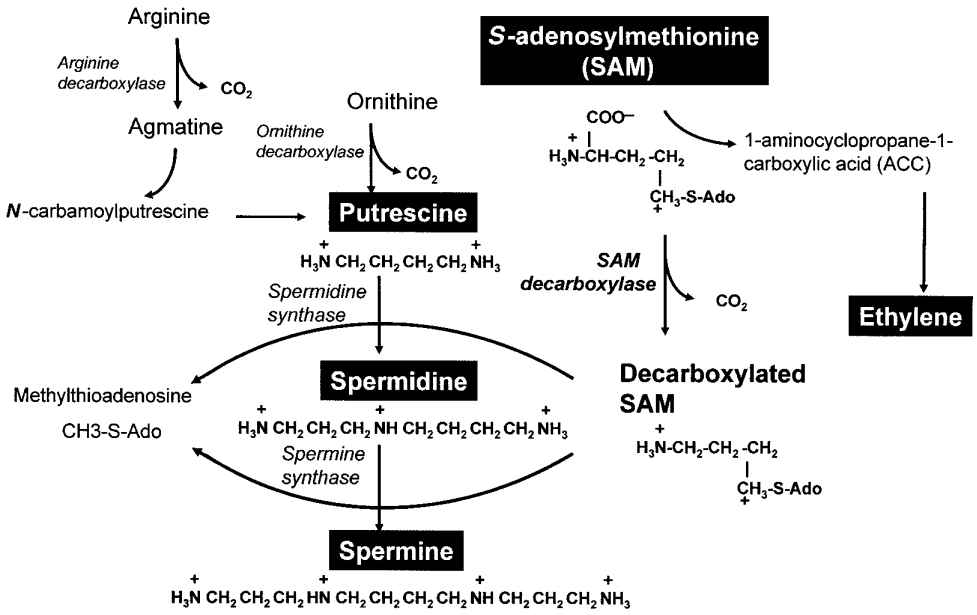

Figure 1. The biosynthetic pathway of polyamines: the intermediates and enzymes (in italics) involved. Also shown is the pathway for ethylene production via formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from *S*-adenosylmethionine (SAM).

polyamines and ethylene may influence specific physiological processes in plants, including fruit ripening[9–12].

Although pharmacological roles of polyamines have been recognized for a long time, direct evidence for their physiological roles in plant metabolism has just begun to be revealed. As indicated by recent studies of constitutively expressed heterologous polyamine genes in tobacco plants, it is possible to manipulate the flux and accumulation of putrescine and higher polyamines[13–15]. In addition, regulated expression of an antisense SAM decarboxylase in potato results in changes in polyamine levels and abnormal phenotypes[16]. Tissue-specific expression of SAM decarboxylase gives rise to smaller tubers without affecting yield[17]. None of these transgenics has been shown to be homozygous for the introduced gene, and the behavior of the plants under field conditions has not been reported.

To gain insight into the *in vivo* function of polyamines in fruit ripening, we introduced the yeast SAMdc gene fused to a fruit-specific promoter into a commercial variety of tomato. Conceptually, selective expression of SAMdc during ripening would result in the accumulation of polyamines and thus influence processes that affect fruit ripening. Fruit from several independent transgenic lines accumulated several-fold higher levels of spermidine and spermine in tomato fruit during ripening. Analysis of the segregating T0 and homozygous (HO) T2–T5 generations of these transgenic plants showed that the increase in polyamine levels is associated with significant increases in the concentration of the antioxidant lycopene and in the quality of the fruit juice, and a substantial increase in the life of the ripened fruit on the vine. The transgenic fruit also produced consistently greater amounts of ethylene during ripening than did the nontransgenics, but the enhanced ethylene production did not modify the temporal regulation of fruit ripening. Taken together, these results provide direct evidence for a physiological role of polyamines in tomato fruit and demonstrate an approach to improving nutritional quality and vine life.

Results

Fruit-specific expression of yeast SAM decarboxylase. A chimeric gene construct containing the yeast SAM decarboxylase (ySAMdc)[18] gene fused to the ripening-regulated E8 promoter[19] was prepared and introduced into tomato plants through *Agrobacterium*-based transformation. Seven independent transgenic tomato plants were selected, screened, and tested for segregation patterns. Progeny were analyzed by PCR analysis of genomic DNA for the presence of the E8-SAMdc gene from a minimum of 20 selfed T0–T5 seeds for each independent transgenic plant. Of the seven transgenic plants selected, three (homozygous lines designated as 556HO, 566HO, and 579HO) followed a normal mendelian segregation pattern for the introduced E8-SAMdc gene. Southern hybridization analysis confirmed the presence of the E8-SAMdc gene in the tomato genome.

The segregation analysis and growth characteristics of the transgenic plants in subsequent generations showed that the accumulation of polyamines under the control of a fruit-specific promoter was not deleterious to plant growth and development, and did not affect the size of the mature fruit. Northern analysis showed that the SAMdc transgene was expressed only in the ripening fruit tissue but not in any other organ of the plant (Fig. 2A).

E8 and ySAMdc transcripts accumulate in ripening fruit. Northern analysis showed that in the transgenic lines, ySAMdc transcript levels appeared at the breaker stage, increased thereafter until the pink stage, and finally decreased at the red stage of ripeness (Fig. 2B). Accumulation patterns of the endogenous E8 transcripts, however, were slightly different in the transgenic plants. In the control, E8 transcript levels increased gradually, most markedly between the turning and pink stages of ripening, whereas in the transgenic line 556HO, E8 transcript levels increased at the turning stage and remained approximately constant throughout ripening. In line SAM579, E8 transcript levels plateaued at the turning and pink stages and declined thereafter.

Spermidine and spermine accumulate in transgenic fruit. The fruit-specific expression of the transgene is consistent with

Table 1. Characteristics of processed juice from control and transgenic fruit expressing the SAMdc gene

Parameter	Wild type	Azygous	Homozygous	
			566HO	579HO
Soluble solids °Brix	4.4 ± 0.4	3.7 ± 0.1	4.1 ± 0.2	4.3 ± 0.0
Acidity % citric acid	3.1 ± 0.2	3.0 ± 0.2	3.0 ± 0.1	3.3 ± 0.2
pH	4.4 ± 0.0	4.4 ± 0.0	4.4 ± 0.1	4.3 ± 0.0
Precipitate weight ratio	9.1 ± 2.7	9.8 ± 2.0	13.0 ± 1.5*	14.5 ± 0.3*
Serum viscosity (s)	80.9 ±11.2	94.9 ± 4.8	106.0 ± 4.4*	119.2 ± 9.8*

Red ripe fruit from these genotypes were processed into juice by the hot break method[32]. The juice was canned and analyzed for °Brix, pH, titratable acidity, serum viscosity, and precipitate weight ratio[32]. *, $P < 0.05$.

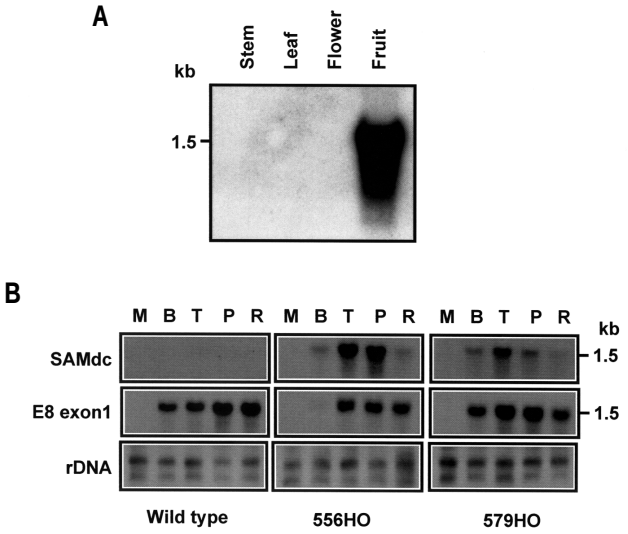

Figure 2. Fruit ripening–specific expression of ySAMdc gene in transgenic tomato. (A) Northern analysis of RNA isolated from stem, leaf, flower, and red ripe fruit of transgenic line 556HO. Each lane contained 25 μg of total RNA. The blot was probed with the 1.2 kb yeast SAMdc fragment. (B) Expression of introduced yeast SAMdc and endogenous E8 genes in fruit of wild-type and transformed tomato plants. Fruit were harvested at mature green (M, full size and firm), breaker (B, orange color on <10% of blossom end of fruit), turning (T, orange to pink color on 10–30% of blossom end), pink (P, pink on 30–60% of fruit surface), and red (R, red color on >90% of fruit surface) stages. Each lane contained 25 μg of total RNA. Hybridization analysis was performed on RNA isolated from fruit harvested at the indicated stage of ripeness. 556HO and 579HO are two transgenic lines homozygous for the transgene.

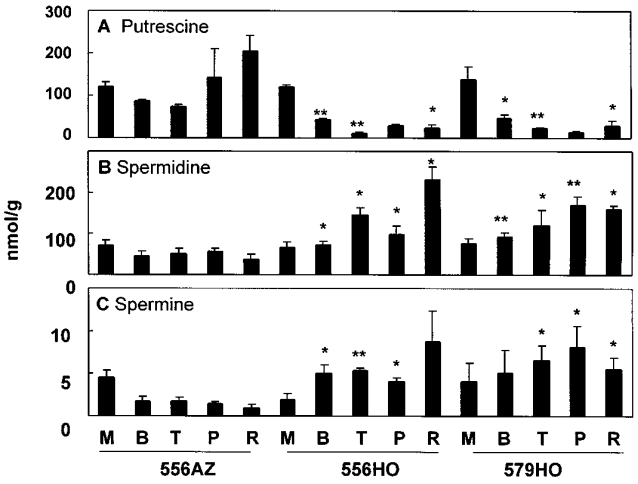

Figure 3. Intracellular levels of putrescine, spermidine, and spermine in pericarp tissue of fruit from wild-type and transgenic lines. Stages of ripeness are described in Figure 2B. Fruit tissue (200 mg) was placed in 800 μl of 5% perchloric acid and stored at –20°C. The samples were frozen and thawed (room temperature) three times and centrifuged at 12,000*g* for 15 min. Heptanediamine was added to the supernatants as an internal standard; they were then dansylated for quantifying polyamines[43]. Values (nmol/g fresh weight) represent averages of data from several independent experiments (n = 4–6) and are shown as mean ± s.e.m.; *, $P < 0.05$; **, $P < 0.01$.

the fact that higher levels of spermidine and spermine were present and maintained in the transgenic fruit during ripening (Fig. 3). Levels of putrescine, spermidine, and spermine gradually decreased in the control fruit as it ripened, except for a precipitous increase in putrescine level only at the pink and red stages. In contrast, spermidine and spermine levels in the fruit from 556HO and 579HO transgenic lines increased gradually during ripening. Clearly, enhanced levels of these polyamines were concomitant with reduced levels of their diamine precursor, putrescine (Fig. 3). These data suggest that the introduced ySAMdc is functional in the tomato fruit and that its expression during ripening brings about compositional changes by increasing the flux of putrescine into spermidine and spermine.

Polyamines act as attenuators of fruit ripening. Analysis of segregating progeny of the three transgenic lines 556, 566, and 579, grown in the field and evaluated for fruit ripening characteristics, showed that ripening was attenuated in the three homozygous lines as compared with azygous (556AZ and 579AZ) and wild-type fruit. A slightly greater attenuation of ripening occurred in the 556HO and 566HO lines as compared with 579HO (Fig. 4A). These data suggest that fruit that maintain high levels of spermidine and spermine have a longer vine life.

Polyamines improve the quality of processed juice. The quality of the processed juice from transgenic fruit was enhanced. The juice from transgenic, azygous, and wild-type fruit had similar levels of soluble solids (°Brix, concentration of soluble solids as a percentage of total fresh weight), pH, and titratable acidity (Table 1). However, both the precipitate weight ratio (PPT) and serum viscosity were increased in the fruit from 566HO and 579HO transgenic lines, as compared with the azygous and parental cultivars. PPT, an indicator of better processing quality of tomatoes[20], was ~50% higher in juice from transgenic fruit as compared with that from the nontransgenic fruit (Table 1). Similarly, the serum viscosity of juice from the transgenic fruit was ~30% and ~50% higher as compared, respectively, with that of juice from azygous and wild-type fruit (Table 1). As PPT represents the fruit components in juice that have not been solubilized by the various enzymatic activities, these results suggest that enhanced polyamine levels may impair fruit disintegration.

Transgenic fruit are enriched in the phytonutrient lycopene. Transgenic ripe tomato fruit described here (Fig. 4B) are deeper red in color than the azygous and wild-type fruit. Like the wild-type fruit, the transgenic fruit were richer in lycopene than in β-carotene. Lycopene content in the transgenic red ripe fruit was increased by twofold and threefold in 556HO and 579HO, respectively, as compared with the wild-type red ripe fruit (Fig. 4C). The higher lycopene content observed in the transgenic fruit was independent of fruit firmness, as the control and transgenic red fruit did not differ significantly in this property.

Ethylene production in transgenic fruit. Fruit from the three transgenic lines (556HO, 566HO, and 579HO) consistently produced ethylene at higher rates than fruit from the azygous line (Fig. 5). Among the three transgenic lines, fruit from 556HO and 579HO produced ethylene at higher rates throughout ripening.

Discussion

We demonstrate that E8-regulated expression of the yeast SAM decarboxylase gene leads to the accumulation of higher polyamines in tomato fruit during ripening. Polyamine accumulation in the fruit, in turn, results in prolonged vine life of the fruit together with an unanticipated twofold to threefold increase in the content of the antioxidant lycopene and enhanced fruit processing quality. These data provide the first evidence of the *in vivo* role of polyamines in the fruit ripening process.

The results showing that red ripe transgenic tomato fruit accumulated 200–300% more lycopene than did the red fruit from the parental line are of special significance, as tomato and its products are an important source of lycopene for human consumption[21]. Lycopene is a biologically important carotenoid, a natural antioxidant whose physical quenching rate constant with singlet oxygen is twice that of

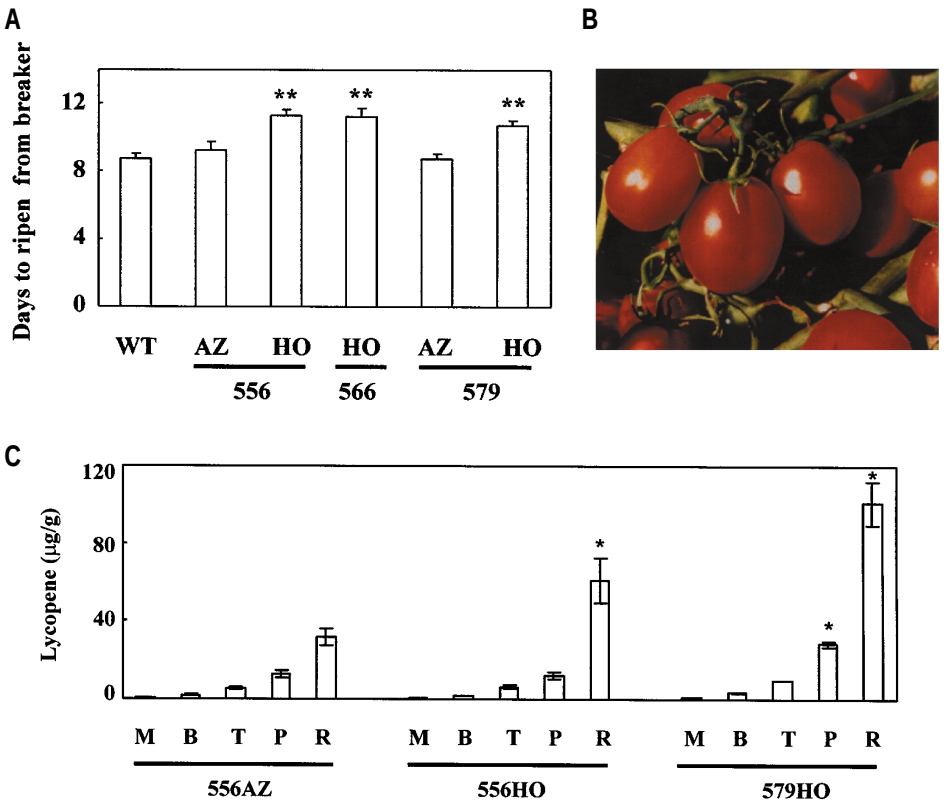

Figure 4. Quality attributes of SAMdc-transgenic tomato fruit. (A) Attenuation of ripening in three transgenic fruit expressing SAMdc as compared with azygous and wild-type controls. Plants homozygous for the introduced SAMdc gene, 556HO, 566HO, and 579HO were grown alongside wild-type (WT) and azygous (AZ) lines (556 and 579) at the Purdue Research Farms. Days from breaker stage to red stage were recorded. Values represent averages of data from several independent seasons (n = 5–10) and are shown as mean ± s.e.m.; *, $P < 0.05$; **, $P < 0.01$. (B) Red fruit from transgenic tomato line 579HO. (C) Enhanced levels of lycopene accumulate at the red stage in transgenic fruit. Stages of ripeness are described in Figure 2B. An acetone/petroleum ether (1:1) extract of tissue was quantified for lycopene content by measuring absorbance at 470 nm (ref. 44). Values (μg/g fresh weight) represent averages of data from several independent seasons (n = 4–6) and are shown as mean ± s.e.m.; *, $P < 0.05$.

β-carotene[22,23]. This characteristic may be responsible for lycopene's ability to mitigate epithelial cancers, including breast cancer and prostate cancer, and coronary heart disease[24,25]. Despite the existence of high-pigment mutants of tomato such as the crimson tomato[26] and of information on several genes encoding carotenoid biosynthetic pathway enzymes[27], very little progress in increasing the lycopene content of tomato fruit had been possible until recently. This is because of the lack of understanding of how plants regulate the biosynthesis of lycopene[27]. Constitutive overexpression of a fruit phytoene synthase (*Psy*) gene, which encodes the first committed enzyme in C_{40} carotenoid biosynthesis, resulted in dwarf tomato plants and ripe transgenic fruit with reduced levels of lycopene as compared with nontransgenic fruit[27]. Through targeted and fruit-specific expression of a bacterial phytoene synthase, however, a 1.8-fold increase in lycopene in transgenic tomato plants has been obtained[28]. This report and our demonstration of an increase (200–300%) in lycopene in tomatoes with a high polyamine content indicate that new genetic engineering approaches may permit increases in phytonutrients in plants. Higher polyamines enhance the accumulation of lycopene in transgenic tomatoes, providing a biological model in which to investigate the molecular regulation of carotenoid biosynthesis. Increased accumulation of carotenoids such as lycopene in tomato fruit is related to the differentiation of chloroplasts into chromoplasts, a developmental process accompanied by redirected and regulated gene transcription and translation that involves genes controlling carotenoid biosynthesis in an ethylene-independent process[29]. How higher polyamines influence these processes should reveal new aspects of chloroplast–chromoplast metabolism.

The increased levels of higher polyamines in the tomato fruit also resulted in increases in precipitate weight ratio and serum viscosity, two parameters that measure the integrity of macromolecules present in fruit. The precipitate weight ratio and efflux viscosity of fruit juice are closely correlated[20]. Also, reduction in pectin breakdown is related to increased tomato juice viscosity[30–33]. The enhanced consistency of the juice processed from the transgenic homozygous fruit was not due to an increase in soluble solids. Taken together, these studies suggest that in the polyamine-rich transgenic tomato, solubilization of the fruit component that decreases juice viscosity is impaired. This hypothesis is consistent with the results showing an increase in the vine life of transgenic fruit. As antioxidants, polyamines may protect against oxidative degradation and membrane damage, resulting in maintenance of homeostasis in fruit cells and a longer ripening phase. This could result in the improved processing attributes (enhanced precipitate weight ratio and serum viscosity) and partially impaired ripening process seen with the polyamine-rich transgenic tomato fruit described here.

SAM is a key intracellular compound with several metabolic functions in a living cell. In flowering plants, SAM is a precursor not only of polyamines, the antisenescence plant growth substances, but also of ethylene biosynthesis. A plant cell, therefore, has the potential to commit the flux of SAM into either polyamine biosynthesis, ethylene biosynthesis, or both. In this context, a question that has been debated in the literature for over two decades is whether or not polyamine and ethylene biosynthetic pathways can function simultaneously in a reproductive plant organ[6,9–11]. Fruit from the transgenic lines described here consistently produced more ethylene than did the nontransgenic control. Thus, it seems that the two metabolic pathways can operate simultaneously *in vivo*, suggesting that the levels of the precursor, SAM, are not limiting for either pathway. Notwithstanding the consistently higher ethylene production rates in the transgenic tomato fruit, the presence of polyamines seems to override the action of ethylene in the ripening of these fruit, by a mechanism still under investigation. In light of the negative-regulation model of ethylene action[34], however, we propose that the accumulation of polyamines may cause qualitative changes in transcriptional activity and may affect ethylene receptor populations or one or more components of the ethylene signal-transduction pathway.

All three transgenic lines produce ethylene at higher rates than the control throughout the ripening process. These results are consistent with published data showing that fruit from plants transformed with an antisense E8 gene have a higher rate of ethylene production[35]. However, the accumulation pattern of endogenous E8 is similar in 579HO and the wild-type control fruit. Although the expression of the

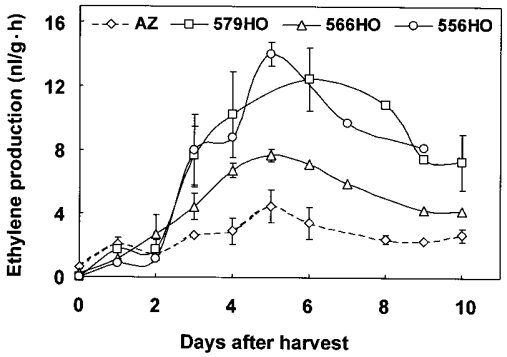

Figure 5. Rates of ethylene production during ripening of fruit from azygous (AZ) and transgenic lines 556HO, 566HO, and 579HO. On day 0, mature green fruit from each line were harvested and taken to the laboratory. On the second day, breaker-stage fruit from each line were selected to align the onset of ripening in each fruit, and thereafter ethylene production by each fruit was measured at the indicated days. Fruit were placed individually in a gas-tight 500 ml glass jar at 20°C for 15 min, after which time a 3 ml sample of head space was analyzed using a gas chromatograph (Varian, model 3700, Palo Alto, CA). Values (nl/h•g fresh weight) on each day represent averages of data from several independent fruit of each line (n = 4–6) and are shown as mean ± s.e.m.

endogenous E8 gene is apparently reduced in transgenic fruit from 556HO, particularly at the breaker stage, as compared with wild-type fruit, our data do not support the hypothesis that higher rates of ethylene production in the transgenic fruit are a result of reduced accumulation of E8 transcripts in these fruit.

Experimental protocol

Transformation of tomato with yeast SAM decarboxylase gene. The 1.2 kb DNA fragment containing the complete coding region of the *S*-adenosylmethionine decarboxylase (SAMdc) gene was amplified by PCR from yeast genomic DNA using primers $P1_{SAMdc}$ (5′-GCGCTGCAGACCATGGCTGT-CACCATAAA-AGAATTGAC) and $P2_{SAMdc}$ (5′-ACAAACTGCAGCTTTT CATATTTTCTTCTGCAA-TTTC), corresponding to the sequence regions of 415–445 and 1605–1572, respectively, of *Spe2* (ref. 18). A *Pst*I restriction site was incorporated at the 5′ end of each primer, and an *Nco*I site was incorporated downstream of the *Pst*I site in the $P1_{SAMdc}$ primer to facilitate cloning into the pCD vector (a gift from Gad Galili) containing a 0.8 kb poly(A)$^+$ sequence. The pCD-SAMdc plasmid was digested with *Nco*I and *Pst*I. Then, the 2 kb DNA fragment containing *Spe2* and the transcription termination sequences was gel purified and cloned into *Nco*I- and *Pst*I-digested pE8mutRN2 plasmid[36] (a gift from Bob Fisher). Chimeric pE8-SAMdc-transcription terminator gene was next inserted into *Eco*RI-digested pMLJ1 vector[37]. The pMLJ-E8-SAMDC plasmid was transferred into the disarmed *Agrobacterium* pGV3850 Ti plasmid[38] to transform a tomato cultivar[39]. The kanamycin-resistant putative transformed plants were grown in a greenhouse. The presence of the full-length transgene and E8-SAMdc junction in T0 and subsequent generations was established by PCR using $P1_{SAMdc}$ and $P2_{SAMdc}$, and E8 promoter (5′-TGTCTCTTTCTTGTTCCCATTTC) and SAMDC (5′-TGGTTGTAGTACCGCACGTC) primers, respectively. Flowers from each transformant were selfed at the T0 and subsequent generations to allow the collection of T0–T5 seeds.

Plant materials and fruit stages. The transgenic and parental commercial processing tomato (*Lycopersicon esculentum* Mill.) cultivar were grown in a greenhouse under natural lighting, in the fields at the Throckmorton Research Farm, Purdue University (W. Lafayette, IN), and at Beltsville Agricultural Research Center Farms (Beltsville, MD).

Nucleic acid isolations. Tomato genomic DNA was extracted from young leaves using a modified CTAB (hexadecyltrimethylammonium bromide) method[40]. DNA and RNA were quantified by measuring absorbance at 260 nm. Nucleic acids were precipitated by the addition of 0.6 volume of absolute ethanol and incubation overnight at –20°C; they were then recovered by centrifugation at 10,000*g* for 30 min. The pellet was resuspended in 10 µl of 50 mM Tris-HCl, 5 mM EDTA, pH 7.5, and then extracted once with phenol/chloroform/isoamyl alcohol (25:24:1 vol/vol/vol) and once with chloroform/isoamyl alcohol (24:1 vol/vol).

Yeast SAMdc and E8 exon 1 probe preparation. The ySAMdc probe was PCR-amplified from the plasmid using primers $P1_{SAMdc}$ and $P2_{SAMdc}$. The E8 exon 1 probe was amplified by PCR using primers FE81 (5′-GGCCGGTGTTAAA-GGACTTGTTGATTCTGG) and RE81 (5′-CCGCATG-GAGTTGAAATTCTTGTAGACT-GGGAGG), based on the first exon of the E8 gene, and tomato genomic DNA as template. The amplified 1.2 kb ySAMdc gene and 0.42 kb of E8 first exon were gel purified (Qiagen, Valencia, CA), randomly labeled with [α-^{32}P]dCTP using the High Prime labeling kit (Boehringer Mannheim, Indianapolis, IN), purified through Nuc-Trap probe purification columns (Stratagene, La Jolla, CA), denatured, and added to the hybridization solution. The ySAMdc probe did not hybridize to the endogenous SAMDC gene (Fig. 2).

Southern and northern analyses. For Southern analysis, 10 µg of tomato DNA was digested for 16 h with 100 units of *Eco*RI, *Bam*HI, or *Pst*I endonucleases (New England Biolabs, Beverly, MA); then another 100 units of the same enzyme was added and digestion was continued for another 3 h. The digested DNA was ethanol precipitated, resuspended in water, and then separated through an 0.8% (wt/vol) agarose gel, with Tris–borate–EDTA as the running buffer, for 16 h at 23 V. The DNA was depurinated in 0.25 N HCl; denatured in 3 M NaCl, 0.5 N NaOH; neutralized in 3 M NaCl, 0.5 M Tris, pH 7.0; soaked in 10× SSC; transferred to a nylon membrane (Zeta-Probe GT, Bio-Rad, Hercules, CA); and cross-linked by ultraviolet radiation at 1,200 µJ (Stratalinker, Stratagene). Tomato stems (1.0 cm segments), leaves, and flowers were brought to the laboratory on ice, frozen in liquid nitrogen, and stored at –80°C. Total RNA was isolated from stem, leaf, flower, and red ripe fruit of transgenic line 556HO (Fig. 2A) by grinding frozen tissue (250 mg) in liquid nitrogen, and then mixed by vortexing with 500 µl of ice-cold denaturing solution (100 mM NaCl, 100 mM Tris, pH 7.5, 1 mM EDTA, and 1% (wt/vol) SDS) and 250 µl phenol. The solution was re-extracted twice with chloroform/isoamyl alcohol (25:1). RNA was precipitated with an equal volume of 4 M lithium acetate at 4°C overnight. The pellet was resuspended in water and 0.1% (vol/vol) sodium acetate was added. The solution was extracted with chloroform/isoamyl alcohol (25:1). RNA was precipitated with ethanol and the pellet resuspended in 10 µl of water. To measure expression of introduced yeast SAMdc and endogenous E8 genes in fruit of wild-type and transformed plants, fruit harvested at mature green, breaker, turning, pink, and red stages (Fig. 2B) were rinsed with distilled water, weighed, monitored for ethylene production, halved, and deseeded. Pericarp tissue was then frozen in liquid nitrogen and stored at –80°C. Total RNA was isolated as described elsewhere[41]. RNA was precipitated by the addition of 0.33 volume of 10 M lithium chloride, followed by a quick freeze in liquid nitrogen and storage overnight at 4°C. RNA was recovered by centrifugation at 12,000*g* for 20 min. The pellet was resuspended in 250 µl of a solution containing 10 mM $CaCl_2$, 10 mM NaCl, 6 mM $MgCl_2$, 40 mM Tris-HCl, pH 7.9, with 10 mM dithiothreitol, and 150 units of RNAsin (Promega, Madison, WI) were added just before use. The solution was incubated with 35 units/ml of RQ1 RNase-free DNase (Promega) at 37°C for 30 min, and then extracted once with phenol/chloroform/isoamyl alcohol (25:24:1 vol/vol/vol) and once with chloroform/isoamyl alcohol (24:1 vol/vol). RNA was precipitated with ethanol and the pellet was resuspended in 0.1 mM EDTA. For northern analysis, 25 µg of total RNA was denatured in a solution containing 50% formamide, 2 M formaldehyde, 0.5 M EDTA, and 0.2 µg/µl of ethidium bromide by heating at 65°C for 15 min and then cooling on ice. The denatured RNA was separated for 2 h at 91 V through a 0.5 M formaldehyde, 1% (wt/vol) agarose gel, with 1 M formaldehyde, 1 M sodium phosphate buffer, pH 6.5, used as the running buffer. An RNA ladder standard (RNA Millennium, Ambion, Austin, TX) was used to estimate the length of the mRNAs. RNA was transferred and cross-linked to a nylon membrane as described above. As a loading control, RNA blots were stripped and reprobed using a tomato 25S rDNA fragment[42]. The blots were prehybridized (4 h) and hybridized (18 h) at 42°C in a solution containing 50% (vol/vol) formamide, 6× SSC, 5× Denhardt's reagent, 0.5% (wt/vol) SDS, and 200 µg/ml denatured sonicated salmon sperm DNA. High stringency washes were performed in 1× SSC, 0.1% (wt/vol) SDS at 42°C for the DNA–DNA hybridization and in 0.1× SSC, 0.1% (wt/vol) SDS at 65°C for the DNA–RNA hybridization blots. Blots were exposed to Kodak X-OMAT XAR-2 film with an intensifying screen at –80°C.

GenBank accession numbers. *Spe2*, M38434; E8, X13437.

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Competing interests statement

The authors declare that they have no competing financial interests.

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